Indian J Med Res 157, June 2023, pp 549-558
DOI: 10.4103/ijmr.IJMR_355_21

Quick Response Code:

Bacteriophages as therapeutic & disinfectant agents to tackle multidrug-resistant *Acinetobacter baumannii*

Nisha Rathor, Tej Bahadur, Chandan Kumar Thakur, Vishwa Deepak Bamola, Bimal Kumar Das & Rama Chaudhry

Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India

Received February 3, 2021

Background & objectives: **Multidrug-resistant (MDR) *Acinetobacter baumannii* is a serious threat for human health worldwide. The studies on agents targeting *A. baumannii* are imperative due to identified *A. baumannii* co-infections in COVID-19. Bacteriophages are promising antibacterial agents against drug-resistant bacteria. This study intended to isolate bacteriophages against MDR *A. baumannii* from the water of river Ganga, to be used potentially as therapeutic and disinfectant particles.**

***Methods*: Acinetobacter phages were isolated from the Ganga water collected from Kanpur and further tested on 50 MDR *A. baumannii* isolates to determine host range. The phages were morphologically characterized by transmission electron microscopy. The disinfectant property of the isolated phages was tested by spraying of bacteriophage cocktail on MDR *A. baumannii* contaminated plastic surface, analyzed by colony-forming unit (CFU) and bioluminescence assay (adenosine triphosphate monitoring).**

***Results*: A total of seven bacteriophages were isolated against MDR *A. baumannii*. The bacteriophages lysed three MDR *A. baumannii* isolates out of 50 tested, showing narrow host range. Electron microscopy revealed hexagonal heads and long tails of bacteriophages, belonging to order *Caudovirales*. The bacteriophage cocktail reduced the MDR *A. baumannii* load efficiently on plastic surface, evidenced by reduction in CFUs and bioluminescence.**

***Interpretation & conclusions*: The findings of this study suggest that the isolated bacteriophages are potential lytic agents for MDR *A. baumannii* clinical isolates, and may be used as potential therapeutic agents as well as disinfectant to combat MDR *A. baumannii* with due consideration to phage host specificity, with further characterization.**

Key words *Acinetobacter baumannii* - antimicrobial - bacteriophage - disinfectant - multidrug resistant

Increasing antimicrobial resistance of pathogenic microorganisms is continually presenting a challenging scenario to human beings and creating a major financial burden on the healthcare system. *Acinetobacter baumannii* has been placed on the top priority by the WHO for research and development to discover novel antibiotics[1] and has been grouped as an ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) pathogen; a

group of drug-resistant nosocomial microorganisms[2]. *A. baumannii* is a fast-growing aerobic bacterium causing a broad range of hospital-acquired infections, bacteraemia, endocarditis, meningitis, septicaemia, ventilator-associated pneumonia, urinary tract infections, wound sepsis, *etc*[3]. It poses a major concern due to the increasing prevalence of drug-resistant strains. Therefore, there is an immediate need to develop a new antibacterial agent to combat this troublesome pathogen.

Bacteriophages are the natural predator of bacteria and considered an attractive and promising class of antibacterial agents against drug-resistant bacterial infections[4]. Bacteriophages have been known as effective antibacterial agents for more than 100 years[4] and can be used as a disinfectant in addition to therapeutic agents[5,6].The bacterial infection can be controlled using a bidirectional approach by the application of bacteriophage as a therapeutic particle as well as a natural disinfectant. There are many reports suggesting the therapeutic and disinfectant potential of bacteriophages against pathogenic bacteria including *A. baumannii* worldwide[7-9]. However, limited studies on this exist from India.

Recent studies have identified *A. baumannii* co-infections[10,11] in COVID-19 cases. Bacterial co-infection may deteriorate the disease condition, especially in ventilator-associated bacterial pneumonia in COVID-19 patients[12], caused by many Gram-negative organisms including *A. baumannii*[13]. Therefore, studies on application of bacteriophage as therapeutic and disinfectant agents become more vital in the context of COVID-19 pandemic.

The present study aimed to, demonstrate the antimicrobial potential of the bacteriophages isolated from river Ganga against multidrug-resistant (MDR) *A. baumannii* as a therapeutic agent as well as a disinfectant on experimental plastic surfaces, for better patient care in the future.

Material & Methods

Antibiotic susceptibility testing: The isolation, identification and antibiotic susceptibility testing of the bacterial isolates were performed at the department of Microbiology (bacteriology section), All India Institute of Medical Sciences (AIIMS), New Delhi, where the samples including blood, pus, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL) and tracheal aspirates (TA) were obtained from different outpatient departments, wards and intensive care units (ICUs), between November 2017 to October 2019, after obaining approval by the Institutional Ethics Committee (Ref No: IEC-484/02.08.2019, RP-54/2019). The samples were processed for bacterial isolation and identification as per standard guidelines. Initially, the samples were inoculated on suitable culture media; blood, pus, ascitic fluid, bile and tissue biopsy on blood agar and MacConkey agar, CSF, sputum, BAL and TA on blood agar, MacConkey agar and chocolate agar, urine on cystine–lactose–electrolyte-deficient agar. The obtained pure culture was identified by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) spectrometry[14].

For each bacterial isolate, the antibiotic susceptibility testing was performed on Mueller–Hinton agar (MHA) using Kirby–Bauer disc diffusion method as per the protocol suggested by Clinical and Laboratory Standards Institute guidelines 2018[15]. The antibiotics used for susceptibility testing included cefotaxime, amikacin, ceftazidime, meropenem, imipenem, ciprofloxacin and piperacillin+tazobactam. The strain was identified as MDR if it was found to be resistant to more than three classes of antibiotics[16]. The bacterial isolates were preserved by making glycerol stocks and kept at −80°C, till further processing.

Collection of water samples & isolation of bacteriophages: Water samples were collected from five different ghats of River Ganga from Kanpur, Uttar Pradesh in sterile containers in December 2018 and transported to AIIMS, New Delhi, at 4°C. The samples were processed for isolation of bacteriophages against MDR *A. baumannii* AIIMS-17 with slight modification[17]. In brief, each water sample was treated with one per cent chloroform (vol/vol) and added to the bacterial culture (log phase) in 1:1 ratio and incubated overnight at 37°C. The suspension was further treated with one per cent chloroform; the supernatant was collected and tested for the presence of bacteriophages by spot assay.

Determination of the antibacterial activity of bacteriophages: To observe the lytic activity of bacteriophages, the spot assay was performed[18]. In brief, bacteriophage preparation (10 µl volume) was dropped onto the bacterial lawn of *A. baumannii* AIIMS-17 and incubated overnight to observe lytic activity. The bacteriophages were further subjected to plaque assay for quantification[19] and presented as plaque-forming units/ml (PFU/ml). For scaling up,

one plaque was cut from the plate, crushed and mixed with the log phase culture of their host and incubated overnight at 37°C. The bacteriophages were harvested after one per cent (v/v) chloroform treatment.

Electron microscopic analysis: The purified bacteriophage particles 1000 µl (10^7-10^8 PFU/ml) were filtered through membrane filters 0.22 µm pore size, and centrifuged at 20800 g for 75 min. The pellet was washed twice with 0.1M ammonium acetate (*p*H 7.0) and negatively stained using phosphotungstic acid (2%, *p*H 7.0)[20]. Briefly, 10 µl of bacteriophage preparation was added on the copper grid, allowed to absorb for 10 min, and further stained with two per cent phosphotungstic acid solution (staining solution) for 15 sec. The stained grid was visualized under transmission electron microscopy (Talos) at Sophisticated Analytical Instrumental Facility, AIIMS, New Delhi. Three measurements of phage head/tail lengths were done using Image J software.

Determination of lysis activity of bacteriophages and host range: To assess the lysis activity of each phage, the active log phage bacterial culture (10^7 colony-forming unit [CFU] /ml) of *A. baumannii* AIIMS-17 was mixed separately (10^7 PFU/ml) with phages. The mixture was incubated at 37°C and the bacterial culture optical density was observed at 600 nm using NanoQuant Infinite M200 Pro Tecan. The bacterial culture without any phage was used as a control. The experiment was done in triplicate.

To determine host range, each phage was subjected to spot assay against 50 MDR isolates of *A. baumannii*.

Thermal stability of bacteriophages: Thermal stability of all seven Acinetobacter phages was investigated at different temperatures. The phage aliquots were incubated at 4, 30, 40 and 60°C for 2 and 6 h, respectively, and enumerated by plaque assay.

Determination of antimicrobial property of bacteriophage cocktail on the plastic surface:

Colony-forming unit (CFU) analysis: To avoid any environmental inhibitory factor and observe the antibacterial property of bacteriophages on MDR *A. baumannii* AIIMS-17 (against which the bacteriophages were isolated) present on surface, the experiment was performed on the plastic surface, in 9 cm diameter petri-plates. The Acinetobacter phage cocktail of all seven isolated phages, AIIMS-Ab-A4, AIIMS-Ab-A5, AIIMS-Ab-A6, AIIMS-Ab-A7, AIIMS-Ab-A8-1, AIIMS-Ab-A8-2 and AIIMS-Ab-A8-3, was prepared by mixing each phage in equal proportion (10^7 PFU/ml).

Bacterial culture of MDR *A. baumannii* AIIMS-17 (200 µl of 1×10^7 CFU/ml) was spread in a petri-plate and allowed to partially dry. The plate was sprayed with 200 µl normal saline or Acinetobacter phage cocktail (10^7 PFU/ml) and covered with lid to avoid drying. At t=0, 2, 4 and 6 h, the swabs were collected (before collecting swab, the surface was allowed to dry) and suspended in 1 ml normal saline, 50 µl volume was spread over MHA plates for CFU analysis.

Adenosine triphosphate (*ATP) monitoring on surface*: To observe the adenosine triphosphate (ATP) level on the surface in the presence and absence of bacteriophage cocktail, the petri-plates were swabbed with MDR *A. baumannii* culture (200 µl of 1×10^7 CFU/ml), sprayed with normal saline or bacteriophage cocktail as mentioned for CFU analysis. The swabs were collected using UXL100 at different time points t=0, 2, 4 and 6 h, and ATP levels were monitored by using 3M™ Clean-Trace™ Hygiene ATP Monitoring system (3M Health Care, St Paul, MN, USA) as per the manufacturer's instruction. The amount of ATP produced; directly proportional to the amount of light emitted by the bioluminescence assay was measured as relative light units (RLUs).

Statistical analysis: The bacteriophages' head *vs.* head and tail *vs.*tail were compared using one-way analysis of variance (ANOVA.) The analysis of change in OD of bacterial culture for lysis assay, temperature stability of phages and CFU and ATP production (bioluminescence) on hard surface were statistically analyzed by GraphPad Prism (version 8.0.2) using repeated measure ANOVA.

Results

Bacterial isolation & identification: A total of 51 MDR clinical isolates of *A. baumannii* including *A. baumannii* complex were isolated from the clinical samples of the study participants. The clinical samples included blood, tracheal aspirate (TA), BAL, pus, CSF, mini BAL, ascitic fluid, bile, pleural fluid, sputum and tissue biopsy (Fig. 1A). The clinical isolates were numbered from AIIMS 1 to AIIMS 51. Each isolate was at least resistant to three classes of antibiotics, therefore, considered MDR. The drug-resistant rates of *A. baumannii* strains

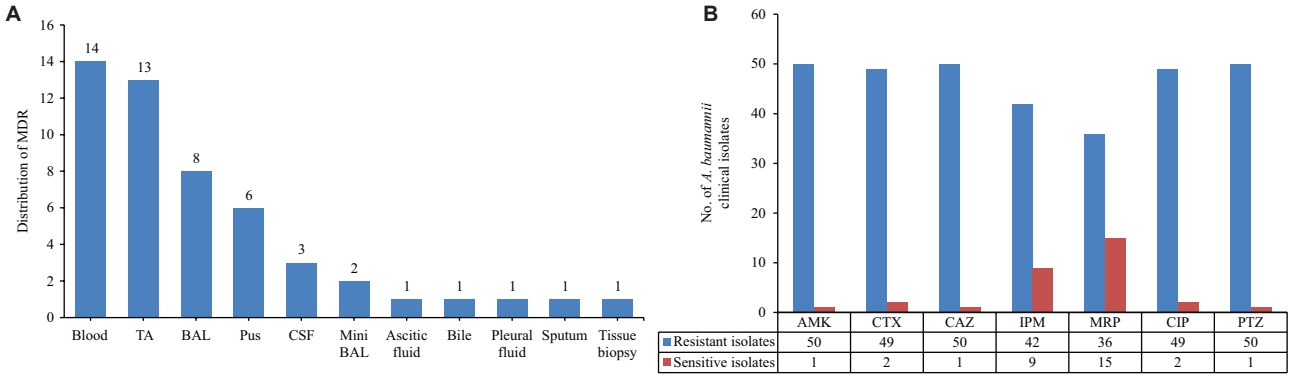

Fig. 1. (**A**) Distribution of 51 MDR *A. baumannii* clinical samples; blood, TA, BAL, Pus, CSF, mini BAL, ascitic fluid, pleural fluid, bile, sputum and tissue biopsy. (**B**) The drug-resistance pattern of 51 MDR *A. baumannii* clinical isolates for different antibiotics; AMK, CTX, CAZ IPM, MRP, CIP, PTZ. MDR, multidrug resistant; TA, tracheal aspirates; CSF, cerebrospinal fluid; AMK, amikacin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; MRP, meropenem; CIP, ciprofloxacin; PTZ, piperacillin +Tazobactam

towards different classes of antibiotics are presented in Figure 1B. The *A. baumannii* isolates showed maximum resistance to amikacin (50/51), ceftazidime (50/51), piperacillin+tazobactam (50/51), cefotaxime (49/51), ciprofloxacin (49/51), imipenem (42/51) and meropenem (36/51).

Isolation of bacteriophages & their lytic activity: A total of seven bacteriophages were isolated from five *Ganga ghats* including *Parmat Ghat* (A4), *Bhairav Ghat* (A5), *Gola Ghat* (A6), *Sarsaiya Ghat* (A7) and *Bhagwatdas Ghat* (A8) from Kanpur, Uttar Pradesh. The nomenclature of the phages was done as follows: AIIMS-two alphabets representing the genus and species of bacterium against which the phage was isolated, followed by the abbreviation of water samples and laboratory number. We could isolate seven bacteriophages: AIIMS-Ab-A4, AIIMS-Ab-A5, AIIMS-Ab-A6, AIIMS-Ab-A7, AIIMS-Ab-A8-1, AIIMS-Ab-A8-2 and AIIMS-Ab-A8-3 against one clinical isolate of *A. baumannii* designated as AIIMS-17. All isolated bacteriophages showed clear lytic activity on performing spot assay (Fig. 2A). The bacteriophages produced circular clear plaques of 1-2 mm in diameter (Fig. 2B).

Microscopic characterization of bacteriophages: The transmission electron microscopy revealed the hexagonal heads and long tail of bacteriophages; therefore, all phages belonged to order *Caudovirales*. The phages AIIMS-Ab-A4, AIIMS-Ab-A5, AIIMS-Ab-A8-1, AIIMS-Ab-A82 and AIIMS-Ab-A83 showed single morphology. The phage AIIMS-Ab-A6 showed a mixture of phages (presented as AIIMS-Ab-A6 (A), AIIMS-Ab-A6 (B) with almost

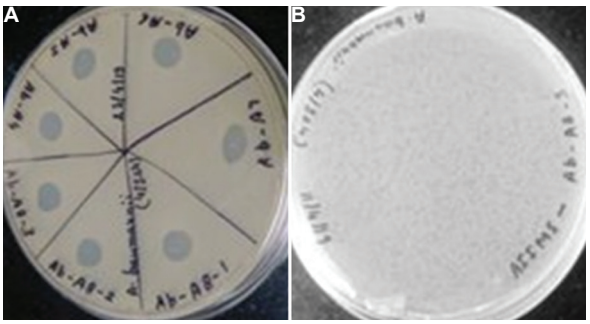

Fig. 2. (**A**) Spot assay showing lysis of *A. baumannii* by different isolated Acinetobacter phages; AIIMS-Ab-A4, AIIMS-Ab-A5, AIIMS-Ab-A6, AIIMS-Ab-A7, AIIMS-Ab-A8-1, AIIMS-Ab-A8-2, and AIIMS-Ab-A8-3. (**B**) A representative picture showing plaques of Acinetobacter phage AIIMS-Ab-A8-3.

similar head and varied tail size (contractile tail), presenting the characteristic feature of *Myoviridae* family of order *Caudovirales*. The morphologies of phages are presented in Figure 3 and measurements are listed in Table I. The head and tail size of phages was compared using one-way ANOVA (Table II) on considering coupled view of head as well as tail, the phages showed significant difference except AIIMS-Ab-A6 (B) and AIIMS-Ab-A8-3.

The lysis curve: To assess the lysis activity of the phages on the host strain, the *A. baumannii* AIIMS-17 host was grown in LB broth and infected by all seven bacteriophages separately, blank served as bacterial control without any phage. The significant reduction in bacterial OD was observed on two hours, followed by a higher reduction up to six hours (Fig. 4) in comparison to a blank ($P<0.01$). Out of all seven used phages, the maximum bacterial growth reduction was observed by AIIMS-Ab-A7 and least by AIIMS-Ab-A8-2.

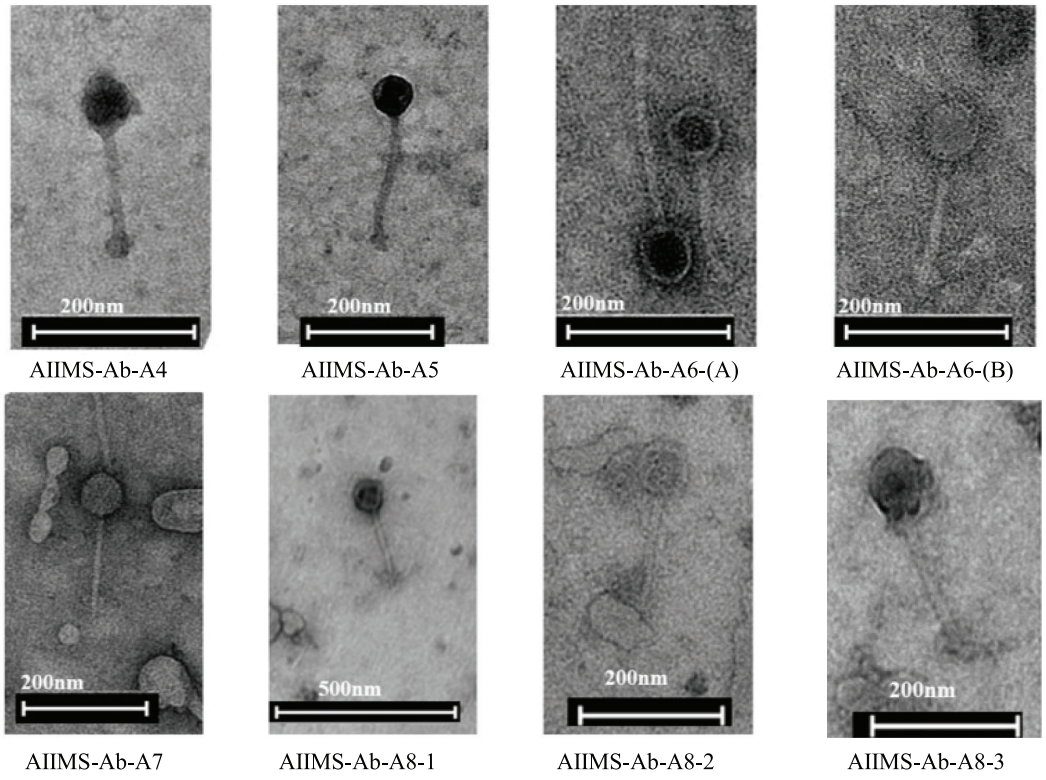

Fig. 3. The transmission electron microscopic view of bacteriophages showing hexagonal heads and long tails.

Table I. Microscopic measurements of bacteriophages active against *Acinetobacter baumannii* obtained from the transmission electron microscopy and measured by Image J software

Bacteriophage	Head size±SD	Tail length±SD
AIIMS-Ab-A4	57.66±1.11	151.74±1.15
AIIMS-Ab-A5	64.08±1.21	192.43±2.21
AIIMS-Ab-A6-(A)	66.03±0.84	208.66±7.02
AIIMS-Ab-A6-(B)	67.36±1.18	154.63±0.53
AIIMS-Ab-A7	64.51±0.69	146.58±2.42
AIIMS-Ab-A8-1	58.94±0.76	142.46±1.83
AIIMS-Ab-A8-2	73.67±0.43	144.92±0.56
AIIMS-Ab-A8-3	69.83±0.52	147.78±2.51

SD, standard deviation; AIIMS, All India Institute of Medical Sciences

Host range analysis: In order to determine the host range of bacteriophages, the isolated bacteriophages were spotted on 51 isolates of MDR *A. baumannii* including the strain AIIMS-17 used as the host for isolation. The lytic activity was observed in only three out of the 50 tested isolates (Table III) in addition to the isolate against which the phages were isolated. The bacterial isolates lysed from the bacteriophages belonged to different clinical samples (Table IV).

Bacteriophages' thermal stability: All bacteriophages remain stable at 4°C for a 6 h exposure. While phage stability varied on temperature exposure of 30°C and 40°C, as presented in Figure 5. The AIIMS-Ab-A8-1 was observed as least stable and AIIMS-Ab-A8-3 showed higher stability at 30 and 40°C. However, at 60°C, no phage could survive even for two hours.

Antibacterial activity of bacteriophage cocktail on the surface: The activity of Acinetobacter phage cocktail over bacterial culture was tested by CFU analysis as well as by the measurement of ATP in terms of RLUs. The CFU analysis showed a significant reduction in the presence of bacteriophages at each time point (P<0.001) (Table V). However, initially, RLUs levels increased, followed by a significant reduction in RLU, produced by phages to bacteria interaction after 4-6 h (Table VI). The initial enhancement in RLUs reflected active lysis of bacterial cells.

Discussion

The MDR *A. baumannii* has become a serious threat to human health worldwide in the recent years[1]. Phage therapy is emerging as a promising approach to treat drug-resistant bacterial infections[21]. Recently, many reports have suggested the potential impact of phage

Table II. Head *vs.* tail comparison using one-way ANOVA

Bacteriophage strain	AIIMS-Ab-A4		AIIMS-Ab-A5		AIIMS-Ab-A6 (A)		AIIMS-Ab-A6 (B)		AIIMS-Ab-A7		AIIMS-Ab-A8-1		AIIMS-Ab-A8-2		AIIMS-Ab-A8-3	
	H	T	H	T	H	T	H	T	H	T	H	T	H	T	H	T
AIIMS-Ab-A4			***	***	***	***	***	NS	***	NS	NS	*	***	NS	***	NS
AIIMS-Ab-A5					NS	***	**	***	NS	***	***	***	***	***	***	***
AIIMS-Ab-A6 (A)							NS	***	NS	***	***	***	***	*	**	***
AIIMS-Ab-A6 (B)									*	NS	***	**	***	*	NS	NS
AIIMS-Ab-A7											***	NS	***	NS	***	NS
AIIMS-Ab-A8-1													***	NS	***	NS
AIIMS-Ab-A8-2															**	NS

P *<0.5; **<0.001; ***<0.001. NS, not significant; H, head of the phage; T, tail of the phage

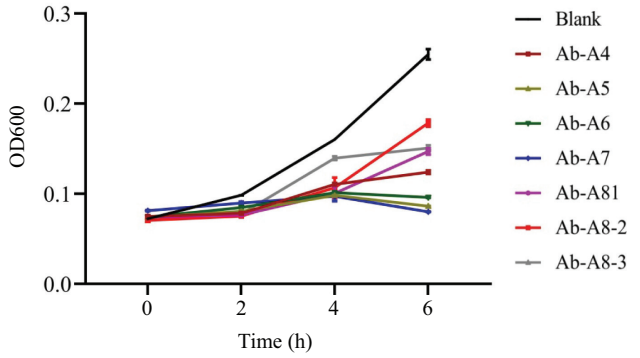

Fig. 4. The lysis curve of MDR *Acinetobacter baumannii* AIIMS-17 infected with different *Acinetobacter* phages, showing a significant reduction in OD in the presence of bacteriophage in comparison to uninfected bacterial culture (Blank). MDR, multidrug resistant

as a therapeutic agent against MDR *A. baumannii*[22,23] as well as a disinfectant[8,9]. The advantage to apply bacteriophages in place of other antimicrobial agents includes; no harm to normal microbiota, auto-dosing of bacteriophages[24]. The safe application of bacteriophages protecting normal microbiota is attributed due to the host specificity of bacteriophages, while the replication ability of phages on its host provides them this auto-dosing property. For therapeutic applications, the phage dose should be maintained for repeated application due to the clearance of phages from the immune system. However, upon application as a disinfectant, auto-dosing plays a role for a better effect[24].

In the present study, seven bacteriophages were isolated, active against MDR *A. baumannii* from the water of the river Ganga. The phages showed a narrow host range on MDR *A. baumannii* clinical isolates. The phages showed effective lysis of the host in liquid culture but varied in their thermal stability. The bacteriophage cocktail effectively lysed the *A. baumannii* on a plastic surface demonstrated by CFU analysis and bioluminescence assay. The TEM showed hexagonal heads and the long tail of all phages.

Bacteriophages are ubiquitous in nature. These are present in rivers, lakes, ponds, sewage, soil, sea and hydrothermal vents[25-27]. The presence of bacteriophages in the Indian rivers Ganga and Yamuna was first discovered in 1896 by Hankin[28]. A recent report[29] also suggested the presence of bacteriophages in the Ganga water against putrefying and pathogenic bacteria. The present study further supports the presence of bacteriophages against MDR *A. baumannii,* attributing the antimicrobial nature of the river Ganga water. The isolated bacteriophages produced clear spots and plaques on MDR *A. baumannii* lawns, indicating their lytic

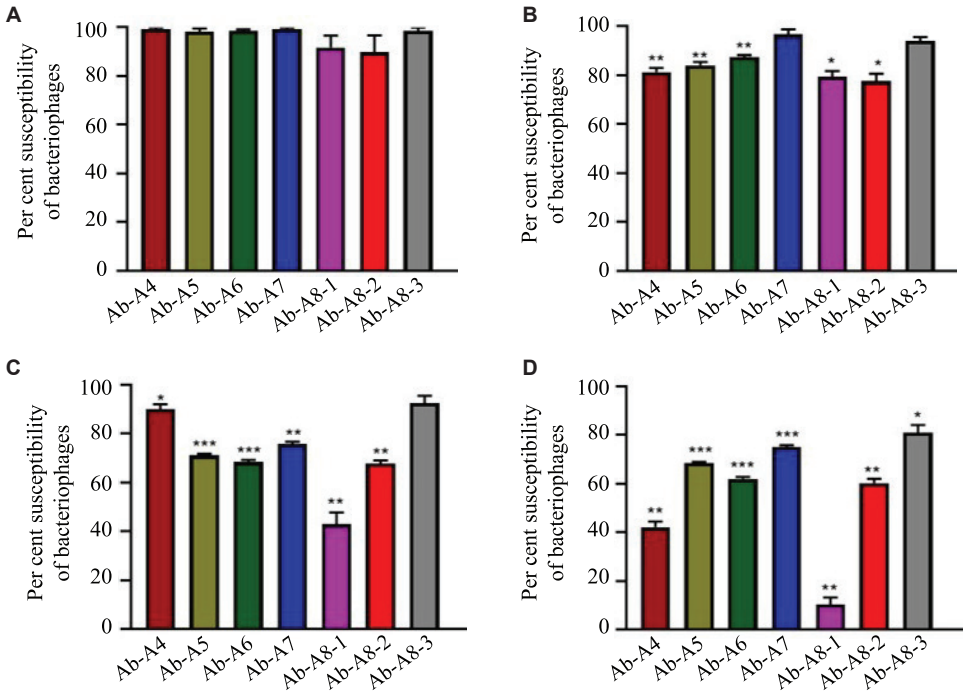

Fig. 5. Thermal stability of different bacteriophages exposed at (**A**) 30°C for two hours, (**B**) 30°C for six hours, (**C**) 40°C for two hours, and (**D**) 40°C for six hours.

Table III. Lytic spectrum of isolated bacteriophages over 50 multidrug-resistant clinical isolates of *Acinetobacter baumannii*

Bacterial isolate number	Bacteriophages						
	AIIMS-Ab-A4	AIIMS-Ab-A5	AIIMS-Ab-A6	AIIMS-Ab-A7	AIIMS-Ab-A8-1	AIIMS-Ab-A8-2	AIIMS-Ab-A8-3
AIIMS-1 to AIIMS-16	−	−	−	−	−	−	−
AIIMS 17	+	+	+	+	+	+	+
AIIMS-18 to AIIMS-30	−	−	−	−	−	−	−
AIIMS-31	+	+	+	+	+	+	+
AIIMS-32	−	−	−	−	−	−	−
AIIMS-33	+	+	+	+	+	+	+
AIIMS-34 to AIIMS 46	−	−	−	−	−	−	−
AIIMS-47	+	+	+	+	+	+	+
AIIMS-47 to AIIMS-51	−	−	−	−	−	−	−

All bacteriophages were isolated against *A. baumannii* AIIMS-17. + indicates lysis of bacteria in presence of phage; − indicates no lysis of bacteria in presence of phage

action, which is in accordance with previous findings[30,31] with a narrow host range of the isolated bacteriophages as reported earlier[32-34]. This phage specificity and narrow host range are probably due to the attachment of the bacteriophages to the specific receptor binding proteins present on host bacterial surface[35].

The disinfectant nature of *A. baumannii* bacteriophages has been successfully demonstrated by others[8,9]. Chen *et al*[8] showed the disinfectant potential of phages on *A. baumannii* on the glass surface. Another study[9] presented the decreased rates of infection caused by carbapenem-resistant

Table IV. Details of multidrug-resistant *A. baumannii* isolates lysed by bacteriophages

Bacterial strain number	Sample type	Antibiotic resistance pattern
AIIMS-17	Tracheal aspirate	AMK, CTX, CAZ, CIP, IPM, PTZ
AIIMS-31	Pus (drain from cardiothoracic and vascular surgery)	AMK, CTX, CAZ, CIP, MRP, PTZ
AIIMS-33	Bronchoalveolar lavage	AMK, CTX, CAZ, CIP, IPM, PTZ
AIIMS-43	Endotracheal aspirate	AMK, CTX, CAZ, CIP, IPM, MRP, PTZ

All bacteriophages have been isolated against *A. baumannii* AIIMS-17. AMK, Amikacin; CTX, Cefotaxime; CAZ, Ceftazidime; CIP, Ciprofloxacin; IPM, Imipenem; MRP, Meropenem; PTZ, Piperacillin + tazobactam

Table V. The bacterial load of multidrug-resistant *A. baumannii* in the presence of *Acinetobacter* phage cocktail on plastic surface (colony forming unit analysis)

Time point (h)	10^5×CFU/ml CFU-Ab±SD	10^5×CFU/ml CFU-AbP±SD	*P* CFU-Ab versus CFU-AbP
t=0	254±19.79	123±16.26	<0.001
t=2	530±14.14	56±3.53	<0.001
t=4	1187±9.8	33±1.41	<0.001
t=6	2306±7.7	25±1.41	<0.001

CFU-Ab: *t*=0 versus *t*=2 *P*<0.001, *t*=2 versus *t*=4 *P*<0.001, *t*=4 versus *t*=6 *P*<0.001; CFU-AbP: *t*=0 versus *t*=2 *P*<0.001, *t*=2 versus *t*=4 *P*<0.05, *t*=4 versus *t*=6 *P*>0.05; CFU-Ab: CFUs of MDR *Acinetobacter baumannii* in presence of normal saline; CFU-AbP: CFUs of MDR *A. baumannii* in presence of *Acinetobacter* phage cocktail. SD, standard deviation; CFU, colony forming unit; MDR, multidrug-resistant

A. baumannii across ICUs in a teaching hospital upon application of daily cleaning practices added with a bacteriophage-containing aerosol against Carbapenem-resistant *A. baumannii*. In this study too, a significant reduction in bacterial load on the application of bacteriophage cocktail over MDR *A. baumannii* (against which, the phages were isolated) was observed. The lysis was also documented by ATP monitoring. The initial interaction of bacteria to phage produced an increase in bioluminescence; indicating the active lysis of bacterial cells[36], followed by reduced bioluminescence in the presence of phage cocktail suggesting a lower bacterial load in comparison to control (bacteria without phage).

The phage characterization including parameters such as morphology, molecular features, pharmacology and immunological aspects are the key components to study and apply them for human benefits[37]. Morphological features are considered an important aspect for bacteriophage classification and characterization. On performing TEM, all bacteriophages showed hexagonal heads and long tails. Therefore, the isolated phages belonged to order *Caudovirales*.

There is growing evidence suggesting the potential of bacteriophage therapy against drug-resistant pathogens. In addition, the bacteriophages may be used as surface disinfectant[6]. The isolation of bacteriophages against *A. baumannii* have been reported earlier for their therapeutic applications. The bacteriophages have been found as an effective antibacterial agent against *A. baumannii* in *Galleria mellonella* larvae as well as the mouse model of acute pneumonia[38]. A successful treatment using bacteriophage was demonstrated against MDR *A. baumannii* for a 68 yr old diabetic patient having necrotizing pancreatitis[22]. In another study, a 77 yr old patient having post-operative MDR *A. baumannii* infection with cerebritis, subdural and epidural empyema was cured by bacteriophage therapy[23].

Recently, *A. baumannii* co-infections have been reported with SARS-CoV-2[10,11], strengthen the urgency of effective and natural antimicrobials against *A. baumannii*. The bacterial viral co-infections may cause miscommunication between the innate and adaptive immunity leading to indirect death in COVID-19 patients. Initially, the innate immune system against high viral load may produce too aggressive response and secretion of inflammatory material into the lungs. The cell debris may nourish the bacterial cells thus causing bacterial co-infections[39]. Bacterial cells may aggravate further innate immune system to add more inflammatory fluid to the lungs and causing severe damage, sepsis and death[40]. On the other hand, the immunosenescence can cause late antibody production, thus delay in recovery in elderly patients[41]. The bacteriophages may be directly applied as aerosol to lyse bacterial co-infections as well as to produce artificial antibodies by phage display[42]. The bacteriophages may also be used to maintain hospital environmental hygiene[9]. Thus, multidirectional application of bacteriophages may lead to rapid recovery and better management of COVID-19 patients.

Table VI. Adenosine triphosphate production (relative light units) on experimentally contaminated plastic surface in the presence of bacteriophage cocktail

Time point (h)	RLU-Ab±SD	RLU-AbP±SD	*P* RLU-Ab *vs*. RLU-AbP
t=0	2469±47.03	2692.218±17.38	<0.05
t=2	12817±394.62	17658.9±162.57	<0.001
t=4	56869±1623	46665.59±1858.51	<0.01
t=6	334299±4629	17525.01±216.38	<0.001

RLU-Ab: RLU produced by MDR *Acinetobacter baumannii* on plastic surface sprayed with normal saline; RLU-AbP: RLU produced by MDR *A. baumannii* on plastic surface sprayed with *Acinetobacter* phage cocktail; RLU-Ab: *t*=0 *vs*. *t*=2 *P*<0.01, *t*=2 *vs*. *t*=4 *P*<0.01, *t*=4 *vs*. *t*=6 *P*<0.001; RLU-AbP: *t*=0 *vs*. *t*=2 *P*<0.001, *t*=2 *vs*. *t*=4 *P*<0.01, *t*=4 *vs*. *t*=6 *P*<0.01. SD, standard deviation; RLU, relative light units

This is a proof of concept study that bacteriophages are innovative methods to deploy to lyse-specific MDR *A. baumannii*. The present study suggests that the strategy to use bacteriophages as a disinfectant may be promising to eliminate specific host with knowledge of the strains present on the surface to be disinfected and its susceptibility to phage collection due to their narrow host range. Therefore, we conclude that the isolated bacteriophages are promising therapeutic and disinfectant agents for specific MDR *A. baumannii*. However, further investigations are required.

***Acknowledgment*:** Authors acknowledge Drs Geetika Yadav, Indian Council of Medical Research, New Delhi; Krishna Khairnar, Environmental Virology Cell, CSIR-National Environmental Engineering Research Institute, Nagpur, for coordinating the study; Gopal Nath, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi for providing training for bacteriophage isolation.

***Financial support & sponsorship*:** This study was financially supported by National Mission of Clean Ganga (NMCG), Ministry of Water Resources and River Rejuvenation (MoWR), Government of India, New Delhi (GKC-01/2016-17/212/NMCG-Research).

***Conflicts of Interest*:** None.

References

1. World Health Organization. *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. Geneva: WHO Press; 2017. p. 1-7.
2. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No ESKAPE. *J Infect Dis* 2008; *197* : 1079-81.
3. Vashist J, Tiwari V, Das R, Kapil A, Rajeswari MR. Analysis of penicillin-binding proteins (PBPs) in carbapenem resistant *Acinetobacter baumannii*. *Indian J Med Res* 2011; *133* : 332-8.
4. Abedon ST, Thomas-Abedon C, Thomas A, Mazure H. Bacteriophage prehistory: Is or is not Hankin, 1896, a phage reference? *Bacteriophage* 2011; *1* : 174-8.
5. Gutiérrez D, Rodríguez-Rubio L, Martínez B, Rodríguez A, García P. Bacteriophages as weapons against bacterial biofilms in the food industry. *Front Microbiol* 2016; *7* : 825.
6. D'Accolti M, Soffritti I, Piffanelli M, Bisi M, Mazzacane S, Caselli E. Efficient removal of hospital pathogens from hard surfaces by a combined use of bacteriophages and probiotics: Potential as sanitizing agents. *Infect Drug Resist* 2018; *11* : 1015-26.
7. El Haddad L, Harb CP, Gebara MA, Stibich MA, Chemaly RF. A systematic and critical review of bacteriophage therapy against multidrug-resistant ESKAPE organisms in humans. *Clin Infect Dis* 2019; *69* : 167-78.
8. Chen LK, Liu YL, Hu A, Chang KC, Lin NT, Lai MJ, *et al.* Potential of bacteriophage ΦAB2 as an environmental biocontrol agent for the control of multidrug-resistant *Acinetobacter baumannii*. *BMC Microbiol* 2013; *13* : 154.
9. Ho YH, Tseng CC, Wang LS, Chen YT, Ho GJ, Lin TY, *et al.* Application of bacteriophage-containing aerosol against nosocomial transmission of carbapenem-resistant *Acinetobacter baumannii* in an Intensive Care Unit. *PLoS One* 2016; *11* : e0168380.
10. Langford BJ, So M, Raybardhan S, Leung V, Westwood D, MacFadden DR, *et al.* Bacterial co-infection and secondary infection in patients with COVID-19: A living rapid review and meta-analysis. *Clin Microbiol Infect* 2020; *26* : 1622-9.
11. Sreenath K, Batra P, Vinayaraj EV, Bhatia R, SaiKiran KV, Singh V, *et al*. Coinfections with other respiratory pathogens among patients with COVID-19. *Microbiol Spectr* 2021; *9* : e00163-21.
12. Póvoa HCC, Chianca GC, Iorio NLPP. COVID-19: An alert to ventilator-associated bacterial pneumonia. *Infect Dis Ther* 2020; *9* : 417-20.
13. Huang Y, Jiao Y, Zhang J, Xu J, Cheng Q, Li Y, *et al.* Microbial etiology and prognostic factors of ventilator-associated pneumonia: A multicenter retrospective study in Shanghai. *Clin Infect Dis* 2018; *67* : S146-52.
14. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, *et al.* Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption

ionization time-of-flight mass spectrometry. *Clin Infect Dis* 2009; *49* : 543-51.

15. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing, 28th ed. CLSI Supplement M100*. Wayne, PA:CLSI ; 2018.
16. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; *18* : 268-81.
17. Ellis EL, Delbrück M. The growth of bacteriophage. *J Gen Physiol* 1939; *22* : 365-84.
18. Kutter E. Phage host range and efficiency of plating. In: *Bacteriophages.* New York, NY, USA: Humana Press; 2009. p. 141-9.
19. Abedon ST, Yin J. Bacteriophage plaques: theory and analysis. *Methods Mol Biol* 2009; *501* : 161-74.
20. Newase S, Kapadnis BP, Shashidhar R. Isolation and genome sequence characterization of bacteriophage vB_SalM_PM10, a Cba120virus, concurrently infecting *Salmonella enterica* serovars typhimurium, typhi, and enteritidis. *Curr Microbiol* 2019; *76* : 86-94.
21. Lin DM, Koskella B, Lin HC. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World J Gastrointes Pharmacol Ther* 2017; *8* : 162-74.
22. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, *et al.* Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob Agents Chemother* 2017; *61* : e00954-17
23. LaVergne S, Hamilton T, Biswas B, Kumaraswamy M, Schooley RT, Wooten D. Phage therapy for a multidrug-resistant *Acinetobacter baumannii* craniectomy site infection. *Open Forum Infect Dis* 2018; *5* : ofy064.
24. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. *Bacteriophage* 2011; *1* : 111-4.
25. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. *Bacteriophage* 2011; *1* : 31-45.
26. Batinovic S, Wassef F, Knowler SA, Rice DTF, Stanton CR, Rose J, *et al.* Bacteriophages in natural and artificial environments. *Pathogens* 2019; *8* : 100.
27. He T, Li H, Zhang X. Deep-sea hydrothermal vent viruses compensate for microbial metabolism in virus-host interactions. *mBio* 2017; *8* : e00893-17.
28. Hankin EH. The bactericidal action of the waters of the Jumna and the Ganges on the cholera vibrio. *Ann Inst Pasteur* 1896; *10* : 511-23.
29. Khairnar K. Ganges: Special at its origin. *J Biol Res (Thessalon)* 2016; *23* : 16.
30. Yang H, Liang L, Lin S, Jia S. Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *BMC Microbiol* 2010; *10* : 131.
31. Yele AB, Thawal ND, Sahu PK, Chopade BA. Novel lytic bacteriophage AB7-IBB1 of *Acinetobacter baumannii*: Isolation, characterization and its effect on biofilm. *Arch Virol* 2012; *157* : 1441-50.
32. Lin NT, Chiou PY, Chang KC, Chen LK, Lai MJ. Isolation and characterization of phi AB2: A novel bacteriophage of *Acinetobacter baumannii*. *Res Microbiol* 2010; *161* : 308-14.
33. Lai MJ, Chang KC, Huang SW, Luo CH, Chiou PY, Wu CC, *et al.* The tail associated protein of *Acinetobacter baumannii* Phage ΦAB6 is the host specificity determinant possessing exopolysaccharide depolymerase activity. *PLoS One* 2016; *11* : e0153361.
34. Ghajavand H, Esfahani BN, Havaei A, Fazeli H, Jafari R, Moghim S. Isolation of bacteriophages against multidrug resistant *Acinetobacter baumannii*. *Res Pharm Sci* 2017; *12* : 373-80.
35. Dunne M, Prokhorov NS, Loessner MJ, Leiman PG. Reprogramming bacteriophage host range: Design principles and strategies for engineering receptor binding proteins. *Curr Opin Biotechnol* 2021; *68* : 272-81.
36. Blasco R, Murphy MJ, Sanders MF, Squirrell DJ. Specific assays for bacteria using phage mediated release of adenylate kinase. *J Appl Microbiol* 1998; *84* : 661-6.
37. Manohar P, Tamhankar AJ, Leptihn S, Ramesh N. Pharmacological and immunological aspects of phage therapy. *Infect Microbes Dis* 2019; *1* : 34-42.
38. Jeon J, Park JH, Yong D. Efficacy of bacteriophage treatment against carbapenem-resistant *Acinetobacter baumannii* in *Galleria mellonella* larvae and a mouse model of acute pneumonia. *BMC Microbiol* 2019; *19* : 70.
39. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: Immunity, inflammation and intervention. *Nat Rev Immunol* 2020; *20* : 363-74.
40. Shi Z, Gewirtz AT. Together forever: Bacterial-viral interactions in infection and immunity. *Viruses* 2018; *10* : 122.
41. Ventura MT, Casciaro M, Gangemi S, Buquicchio R. Immunosenescence in aging: Between immune cells depletion and cytokines up-regulation. *Clin Mol Allergy* 2017; *15* : 21.
42. Wojewodzic MW. Bacteriophages could be a potential game changer in the trajectory of coronavirus disease (COVID-19). *Phage (New Rochelle)* 2020; *1* : 60-5.

For correspondence: Dr Rama Chaudhry, Department of Microbiology, All India Institute of Medical Sciences, New Delhi 110 029, India
e-mail: drramach@gmail.com